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13. ABSTRACT (Maximum 200 Words)

Mutations in the tumor suppressor gene, BRCA1, account for 45% of families with a high incidence of breast cancer and the majority of families with high incidences of both breast and ovarian cancers. Recent data has shown BRCA1 to be associated to be associated with a human SWI/SNF complex, serving to link breast cancer to chromatin remodeling (3). Current evidence points to the idea that BRCA1 works through SWI/SNF; therefore, a molecular understanding of the SWI/SNF complex and other human chromatin remodeling complexes will offer insight into the biology of BRCA1. The central catalytic ATPase subunit of SWI/SNF is BRG1; the central catalytic subunit of a related human chromatin-remodeling complex, NURF, is SNF2H. Initially, crystallization and X-ray structural determination of the core ATPase domain, in addition to the full-length proteins was undertaken with much difficulty. Currently crystals have been grown for the ATPase domain of Brg1 and crystallization trials are underway for the related full-length human protein, SNF2H. Initial purification and expression of a SWI/SNF functional core and the SNF2H containing NURF complex were unsuccessful. The conserved core ATPase domain was identified in two organisms that are more primitive. A recombinant homologous ATPase domain from both organisms was expressed, purified, and three-dimensional crystals were grown. Optimization and refinement of these initial crystals was underway to obtain X-ray diffraction quality crystals for structural determination of these homologous archaeal ATPase domains, but were derailed by a recently published X-ray structure of the *Sulfolobus sulfataricus* SWI2/SNF2 ATPase core and its complex with DNA (18).

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Table of Contents

Introduction :

Breast cancer is the most common malignancy in women in the Western world (1). BRCA1 is a tumor suppressor gene linked to familial breast and ovarian cancers (4, 5). Current evidence has led to the hypothesis that BRCA1 may function as a transcriptional regulator (2). The strong correlation of cancer-associated mutations and its loss of function phenotype in recent experiments, strengthen the idea that the role of BRCA1 in transcription is physiologically relevant during the development of disease (2). Furthermore, BRCA1 has been linked to chromatin remodeling complexes, specifically through the stable association of the BRCA1 protein with BRG1, the catalytic subunit of the SWI/SNF chromatin-remodeling complex (3). The recent evidence that implicates BRCA1 as a component of a human SWI/SNF chromatin-remodeling complex implicates chromatin-remodeling complexes in the pathogenesis of breast and ovarian cancers (3).

Chromatin remodeling is associated with gene expression and the subsequent assembly in an energy dependent manner of both activating and repressive proteins in the nucleosomal infrastructure. The mistargeting of these enzymes contributes to human developmental abnormalities and tumorigenesis, illustrating the role of chromatin remodeling complexes in human disease (6). Regulation of gene expression occurs in the context of chromatin whose structure inhibits transcription at various levels, including activator binding, preinitiation complex formation, and transcriptional elongation (7). In the past five years, numerous ATP-dependent chromatin-remodeling complexes have been isolated and characterized.

All of the ATP-dependent chromatin-remodeling complexes contain an ATPase subunit that belongs to the SNF2 superfamily of proteins. Enzymes that resemble SWI2/SNF2 in their ATPase domains form a distinct subfamily within the family of nucleic-acid-stimulated DEAD/H ATPases (8). The SWI2/SNF2 subfamily can be further divided into at least four groups of enzymes, according to the domains flanking their ATPase domains, biochemical properties, and mechanisms of nucleosome remodeling. BRCA1 has been found to be associated with a human SWI/SNF (hSWI/SNF) complex, which contains either BRG1 or hBRM as the central ATPase (8). The SWI/SNF complexes contain eight or more subunits, and each contains a DNA-dependent ATPase homologous to that of yeast SWI2/SNF2. It has been demonstrated that an efficient ATP-dependent remodeling complex can be constituted using a subset of the proteins found in the human SWI/SNF complex, specifically BRG1, BAF170, BAF155, and INI1 with BRG1 as the central ATPase (8).

A second class of ATP-dependent chromatin remodeling complexes is the ISWI-based complexes, which contain 2-4 subunits including the central ATPase. The ISWI group includes yeast ISWI1 and ISWI2, *Drosophila* NURF, CHRAC, and ACF, and human WCRF/ACF, CHRAC, and RSF complexes (9). A four-subunit human NURF complex with the protein SNF2H as the central ATPase has recently been identified (data not published). It has only recently been demonstrated that the central ATPase subunits of both SWI/SNF and ISWI complexes can alter chromatin structure in the absence of any remaining subunits (10).

Taken together, chromatin-remodeling complexes, such as SWI/SNF and NURF play critical roles in gene regulation. Current evidence points to the idea that BRCA1 works through SWI/SNF, SWI/SNF and NURF complexes are related functionally and compositionally, and therefore a molecular understanding of the SWI/SNF and NURF complexes will offer insight into the biology of BRCA1.

Body:

The majority of work completed within the past 3 years has been predominantly focused on the first task outlined in the approved statement of work: *the determination of the X-ray crystal structure of the ATPase domain of BRG1*. The crystallization of this catalytically active chromatin-remodeling enzyme has been difficult. Bacterial expression, multiple attempted purification schemes, and refolding experiments of the ATPase catalytic core domain of BRG1 failed to produce significant amounts of pure, soluble protein for crystallization. A pure, soluble, recombinant ATPase domain of BRG1 was finally prepared with insect cells using a baculovirus expression system and subsequently purified by FLAG-affinity chromatography coupled with gel filtration. Crystallization factorial screens (11, 12) utilizing the hanging drop method were set up with protein alone, in the presence of ATP, or in the presence of a stable non-hydrolyzable analogue, ATP-γ-S at 20 °C and 4 °C. No crystals were obtained, but the rate-limiting step at this time was not crystallization, but the amount of purified protein that could be produced. In this past year, the problem was overcome as a direct result of collaboration with the Kingston group at Harvard Medical School, who had the facilities and knowledge to scale up the insect cell expression system. They supplied the purified ATPase domain of Brg1 (residues 728 to 1386), that was used for subsequent crystallization studies. Recently, the ATPase domain of Brg1 has been crystallized both with and without ATP-γ-S (Fig. 1), but the crystals have not been tested for diffraction yet. Refinement of the original crystallization condition is underway to obtain larger, diffraction quality crystals.

Another approach undertaken in the attempted realization of the first task outlined in the approved statement of work focused upon the crystallization of the full-length human SNF2H protein. SNF2H is the central ATPase of the ISWI class of ATP-dependent chromatin remodeling enzymes that contains a conserved catalytic ATPase core domain with Brg1. Initial expression and purification utilizing a baculovirus insect cell expression system was promising, but again protein aggregation and limited protein expression levels precluded any crystallization experiments. This problem was alleviated by the same collaboration with the Kingston group, in which they supplied purified SNF2H from a larger scale baculovirus expression system. Initial crystallization factorial screens (11, 12) utilizing the hanging drop method were set up with protein alone, or in the presence of ATP-γ-S at both 20 °C and 4 °C. No crystals have yet to be observed.

An alternative approach to the completion of the first task involved the identification of a conserved ATPase domain located in a more primitive evolutionary state. The ATPase domain of BRG1 is highly conserved and two archaeal homologues were identified using a BLASTP search in conjunction with a SMART (Small Molecular Architecture Research Tool) analysis (13, 14). The top three matches were: a SWI/SNF helicase from *methanosarcina mazei* (Accession number: Q8PWU7), a SNF2 helicase from *methanosarcina acetivorans* (Q8TU84), and two hypothetical proteins SSO1653 and SSO1655, that together form a conserved BRG1-like ATPase domain, from the thermostable archaea, *sulfolobus solfataricus* (Q97XQ5 and Q97XQ7). The proteins from *methanosarcina mazei,* denoted **MOATP** (for abbreviation purposes only), and from *sulfolobus solfataricus,* denoted **SSOATP,** were chosen as viable targets because both of their genomic DNA were commercially available.

The ATPase domain of MOATP and SSOATP were identified from sequence homology comparison with BRG1 (Fig. 2). Constructs for the ATPase domain of MOATP were designed to include amino acid residues 550 to 1089 with an N-terminal cleavable histidine tag spanning the previously defined ATPase domain. The protein was bacterially expressed, purified to homogeneity and crystallization trials were undertaken. The recombinant ATPase domain from MOATP was assayed for ATPase activity and chromatin remodeling ability in two experiments. The recombinant

ATPase domain of MOATP exhibits intrinsic DNA-stimulated activity (Fig. 3), but not nucleosome-stimulated ATPase activity as compared to recombinant full-length SNF2H, a human homologue to BRG1 (15). The recombinant ATPase domain does not display chromatin-remodeling activity as evidenced by a negative result on a Sal I nucleosome accessibility assay (16). Initial crystallization (11, 12) factorial screens utilizing the hanging drop method were set up at 20 °C with and without ATP-γ-S, and after 4 to 5 weeks, small, needle-like crystals were observed. These crystals were not of diffraction quality and refinement yielded only negative results. They could not be improved by biochemical techniques in which new constructs were designed (a FLAG tagged construct to improve purity), by protease cleavage of the original histidine affinity tag, nor could they be rimproved by known crystallographic refinement methods.

The difficulty encountered with the purification and crystallization of the initial archaeal homologue of Brg1, led to the design of a single construct for the ATPase domain of SSOATP spanning residues 227 to 788 of hypothetical protein SSO1653 and residues 6 to 124 of hypothetical protein SSO1655 with an N-terminal cleavable histidine tag spanning the previously defined ATPase domain. The protein was purified using affinity, ion exchange, and size exclusion chromatography, and initial crystallization trials yielded small crystals (Fig 4). The enzymatic ATPase activity of this archaeal construct was similar to that of the previously crystallized archaeal homologue, MOATP. The crystal diffracted weakly to only 8 – 10 Å. Refinement of this crystal form and the identification of a shorter construct from proteolysis provided further evidence that crystallization of this archaeal homologue was possible. This foresight did prove to be true, when just recently, another group published the crystal structure of the *Sulfolobus solfataricus* SWI2/SNF2 ATPase core, which corresponded to our tryptic digest from the proteolysis experiments (18).

The second task defined under the initial statement of work was to *determine the structure of a SWI/SNF complex containing the catalytic ATPase*. The preparation and purification of a functional BRG1 core SWI/SNF sub complex, identified by Kingston (17) has been attempted unsuccessfully. BRG1 is homologous to SNF2H, the catalytic ATPase of the human ISWI complex, NURF, first identified by the Shiekhattar group at the Wistar Institute (15). The NURF complex is composed of the catalytic ATPase SNF2H and two other auxiliary subunits, BPTF and Rbap48. Co-expression of the three proteins and subsequent FLAG affinity purification does not yield a stable recombinant complex, because BPTF is proteolytically digested during expression in the Sf9 insect cells.

Figures:

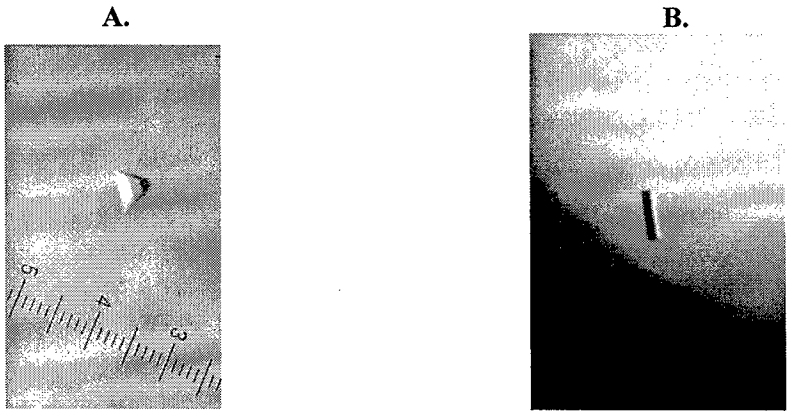

Figure 1: Initial crystals of the ATPase domain of human Brg1. (A) Brg1 crystals from the following condition : 100 mM Imidazole, pH 8.0 and 1.0 M $(NH4)_2HPO_4$ at 4 °C. (B) Brg1 bound to ATP-γ-S crystals grown in the same condition as in (A), but at 20 °C.

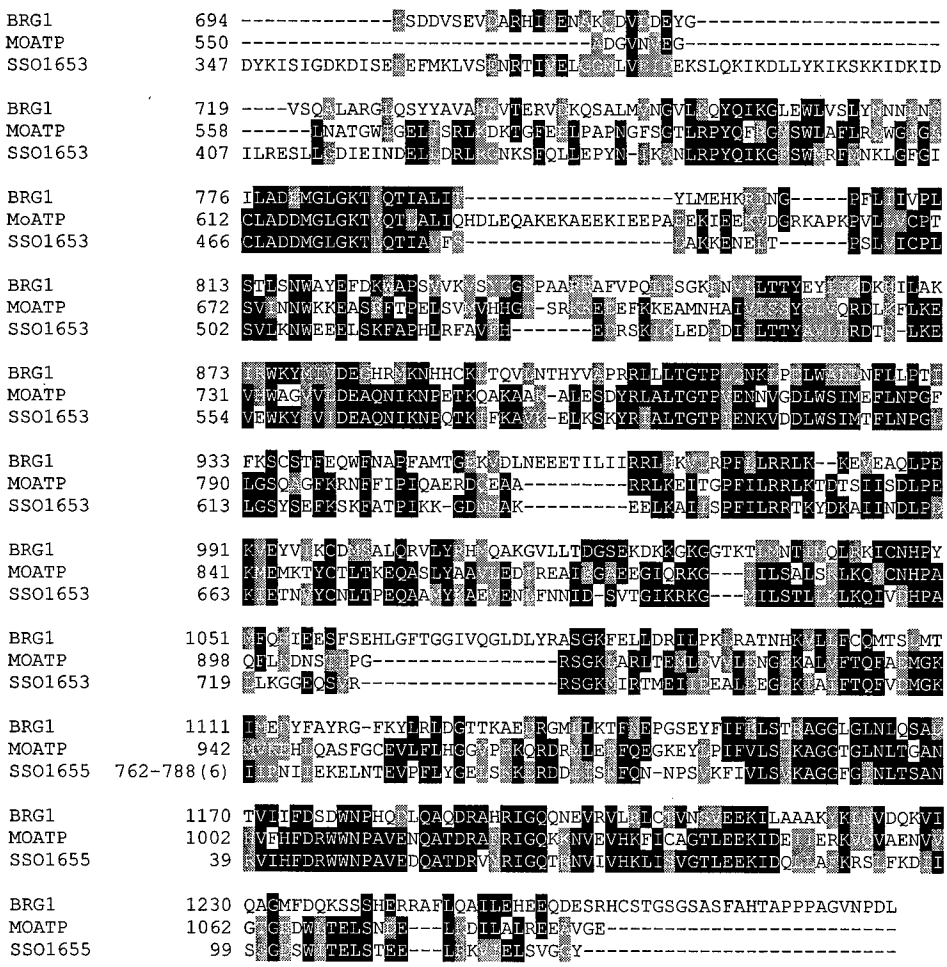

Figure 2: Sequence Alignment of the ATPase domain of BRG1 with the archaeal homologues from *methanosarcina mazei* and *sulfolobus solfataricus.*

Figures (con't)

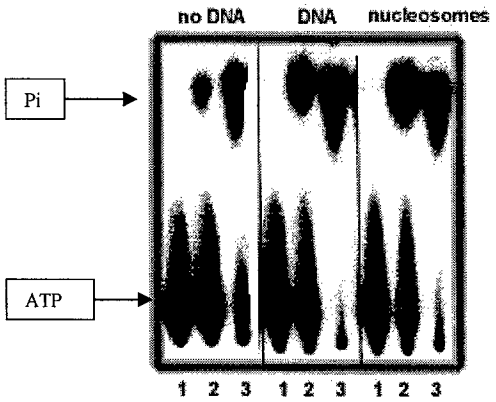

Figure 3: MOATP exhibits DNA-stimulated ATPase activitiy. # 1 denotes buffer alone (negative control; # 2 is MOATP; # 3 is recombinant full-length SNF2H (positive control).

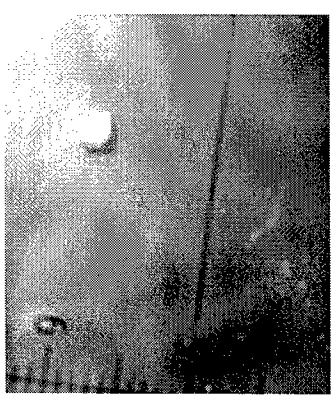

Figure 4: Initial crystals of SSATP from the following condition: 0.1 M Imidazole, pH 8.0; 1.0 M $(NH4)_2HPO_4$; 0.2 M NaCl, at 19 °C.

Key Accomplishments:

- Preliminary three-dimensional crystals for the ATPase domain of Brg1.
- Bacterial expression and purification of an extended ATPase core domain from *methanosarcina mazei* spanning residues 550 to 1089.
- This extended ATPase core domain from *methanosarcina mazei* displays intrinsic DNA-dependent ATPase activity as evidenced by a performed ATPase activity assay.
- Factorial crystallization screens of the purified extended ATPase domain from *methanosarcina mazei* yielded preliminary needle-like crystals.
- Bacterial expression and purification of an extended ATPase domain from the thermostable archaea, *sulfolobus solfataricus*, with similar ATPase enzymatic activity as MOATP.
- Factorial crystallization screens of the purified extended ATPase domain from *sulfolobus solfataricus* yielded small three-dimensional crystals with weak diffraction. Refinement of theses crystals in addition to the isolation of a proteolytically stable cleavage product and subsequent crystallization was pre-empted by a published structure of this SSOATP construct that was not ours.
- Expression of the SNF2H-containing NURF complex in insect cells using a baculovirus system. SNF2H is a related ATPase associated with the ISWI family of chromatin remodeling complexes. Factorial crystallization screens of the full-length human SNF2H have only recently been undertaken; crystal formation has yet to be observed.

Conclusions:

The determination of the crystal structure of the ATPase domain of BRG1 and the full-length protein has proved difficult. Neither the ATPase domain nor the full-length protein could be expressed and purified in significant amounts for crystallization. Numerous purification and expression strategies were employed to no avail. Eventually, suitable expression and purification levels for the ATPase domain of Brg1 were obtained in a collaborative effort with the Kingston group at Harvard. Initial factorial crystallization screens have yielded preliminary three-dimensional crystals. The purification and crystallization of a related ATPase family member, SNF2H, was also attempted under the same collaborative effort. Initial crystallization trials have not resulted in any diffraction quality crystals, yet. Another approach that was employed to address the problems with expression and purification, was to express these ATPase proteins within a context that lends stability and allows manipulation conducive to crystallization experiments. For this reason, expression and purification of the functional core of SWI/SNF, and a SNF2H containing NURF complex was attempted. Initial attempts at expression and purification of these complexes were unsuccessful.

An alternative attempt to determine the crystal structure of the ATPase domain of BRG1 has been undertaken with a promising degree of success. Two archaeal homologues of the extended ATPase core domain of BRG1 have been identified, and cloned into T7 expression vectors. The ATPase domain from the protein MOATP, endogenous to the archaea *methanosarcina mazei*, has been expressed and purified by a combination of affinity, ion exchange, and size-exclusion chromatography. Initial crystallization factorial screens yielded small-needle like crystals in more than one crystallization condition, suggesting that diffraction-quality crystals can be obtained in the near future. ATPases assays were performed with the recombinant ATPase domain from BRG1, and the recombinant protein demonstrated intrinsic DNA-dependent ATPase activity. The second archaeal homologue from *sulfolobus solfataricus* was also expressed, purified, and assayed for ATPase activity. It was also crystallized but before refinement could be completed, a structure of this homologue was just recently published (18).

Abbreviations and Acronyms:

ATP: adenosine tri-phosphate

BL21 (DE3)*: competent bacterial cell type

BRCA1: tumor suppressor gene and translated protein

BRG1: brahma-related protein-1

FLAG: affinity tag

INI-I11: human HeLa cell line

ISWI: imitation switch

RSC: remodel the structure of chromatin

SF9: insect cell-line used in baculovirus expression system.

SDS-PAGE: Sodium dodecyl sulfate polyacrylimide gel electrophoresis

SWI/SNF: switch/sucrose non-fermenting

SNF2H: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5. The gene name is SMARCA5.

HBRM: human brahma protein

MOATP: an arbitrary abbreviation for a SWI/SNF helicase protein from *methanosarcina mazei.*

SSOATP: an arbitrary abbreviation for a hypothetical SWI/SNF helicase protein from *sulfolobus solfataricus.*

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